

CLAIMS

1. A method of in vitro amplification of nucleic acid using
5 amplification primers in a manner known per se, in amplification
reactions such as PCR, LCR or NASBA, wherein a pair of primers is used
comprising oligonucleotide sequences sufficiently complementary to a part
of the Direct Repeat sequence of a bacterium other than a microorganism
10 belonging to the M tuberculosis complex of microorganisms for
hybridisation to a Direct Repeat to occur and subsequently elongation of
the hybridised primer to take place, said primers being such that
elongation in the amplification reaction occurs for one primer in the 5'
Direction and for the other primer in the 3' Direction, wherein the
15 Direct Repeat is a sequence with a length between 20-50 base pairs which
occurs 5-60 times in a region of the bacterial genome, whereby the Direct
Repeat sequences are separated by spacer sequences with a length of
between 20-50 nucleotides, said spacer sequences being non repetitive.

2. A method according to claim 1 wherein the Direct Repeat
20 sequence is obtainable from screening a genomic bacterial nucleic acid
sequence using the programme Patscan wherein the Direct Repeat is
designated p1 with a length between 20-50 basepairs then p1 is sought 20-
50 basepairs downstream of p1 as the pattern
p1=(20..50)(20..50)p1(20..50)p1 or a variant thereof wherein the ranges
25 of the nucleotide lengths are shorter and wherein the frequency of
occurrence of the Direct Repeat can vary between 5 and 60.

3. A method according to claim 1 or 2 wherein the Direct Repeat
30 has a length between 30-40 base pairs and the spacer has a length of 35-
45 base pairs.

4. A method according to any of the preceding claims wherein the
Direct Repeat has a terminus exhibiting at least 3 out of 5 nucleotides
identical with the sequence GAAAC, preferably 4, said termini for example
35 being selected from GAAAC, GAAXXC GAACTC, GXAAC, GCAAC, GAAA, GAAXC,
GAAGC, AAAC.

5. A method according to any of the preceding claims wherein the
Direct Repeat terminates with GAACTC, ATACAC, AAAACT, TTGCAA, GGAAAC,

Revised by Pat 34

TGAAAC, TGAAGC, TGGAAA, TTAAAC, TGAAAT or TTCAAC.

6. A method according to any of the preceding claims wherein the Direct Repeat has stretches of 3-4 identical bases.

7. A method according to any of the preceding claims wherein the Direct Repeat has a sequence such that it is not prone to loop formation or any other obvious secondary structure.

8. A method according to any of the preceding claims wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative bacteria of Groups 4 and 5 of Bergeys Determinative Manual of Bacteriology ninth edition, in particular the families Enterobacteriaceae, Pasteurellaceae and Vibrionaceae of Group 5, most specifically the Enterobacteriaceae and the Gram positive bacteria of Group 17.

9. A method according to any of the preceding claims wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative bacteria of Bergeys Determinative Manual of Bacteriology ninth edition of the genera Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas, Bordetella, Staphylococcus, Streptococcus and Acinetobacter.

10. A method according to any of the preceding claims, wherein said primers have oligonucleotide sequences complementary to non overlapping parts of the Direct Repeat sequence and such that the elongation reactions from each primer can occur without hindrance of the other when both primers hybridise to the same Direct Repeat and undergo elongation.

11. A method according to any of the preceding claims, wherein one primer DRa is capable of elongation in the 5' Direction and the other primer DRb is capable of elongation in the 3' Direction and DRa is complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary.

12. A method of detection of a bacterium, said bacterium not belonging to the M tuberculosis complex of microorganisms said method

comprising

- 1) amplifying nucleic acid from a sample with the method according to any of the preceding claims, followed by
- 2) carrying out a hybridisation test in a manner known per se, wherein the amplification product is hybridised to an oligonucleotide probe or a plurality of different oligonucleotide probes, each oligonucleotide being sufficiently homologous to a part of a spacer of the Direct Region of the bacterium to be determined for hybridisation to occur to amplified product if such spacer nucleic acid was present in the sample prior to amplification, said hybridisation step optionally being carried out without prior electrophoresis or separation of the amplified product.
- 3) detecting any hybridised products in a manner known per se.

13. A method according to claim 12, wherein the hybridisation test is carried out using a number of oligonucleotide probes, said number comprising at least a number of oligonucleotides probes specific for the total spectrum of bacteria it is desired to detect.

14. A method according to claim 12 or 13, wherein the oligonucleotide probe is at least ten oligonucleotides long and is a sequence complementary to a sequence selected from any of the spacer sequences of the Direct Repeat region of the bacterium to be determined or is a sequence complementary to fragments or derivatives of said spacer sequences, said oligonucleotide probe being capable of hybridising to such a spacer sequence and comprising at least ten consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology with such a spacer sequence.

15. A method according to any of claims 12-14 wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative bacteria of Groups 4 and 5 of Bergeys Determinative Manual of Bacteriology ninth edition, in particular the families Enterobacteriaceae, Pasteurellaceae and Vibrionaceae of Group 5, most specifically the Enterobacteriaceae and the Gram positive bacteria of Group 17.

16. A method according to any of claims 12-15 wherein the bacterium

is a pathogenic bacterium selected from the group of Gram negative bacteria of Bergeys Determinative Manual of Bacteriology ninth edition of the genera Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas and Bordetella and the group of Gram positive bacterial genera Staphylococcus and Streptococcus as target for the differentiation method.

17. A method for differentiating the type of bacterium in a sample, said bacterium not belonging to the M. tuberculosis complex, said method comprising carrying out the method according to any of claims 12-16, followed by comparison of the hybridisation pattern obtained with a reference.

18. A method according to claim 17, wherein the reference is the hybridisation pattern obtained with one or more known strains of the bacterium to be determined in analogous manner.

19. A method according to claim 17 or 18 wherein the reference is a source providing a list of spacer sequences and sources thereof, such as a data bank.

20. A pair of primers wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism E. coli for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular a Sequence from Table II and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence.

21. Primer pair according to claim 21, comprising one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to

a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of *E. coli*.

5 22. A pair of primers wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism *S. typhimurium* for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being
10 such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular the *E. coli* Sequence of Table II
15 and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence.

23. Primer pair according to claim 22, comprising one primer DRa
20 capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of *S. typhimurium*.

25 24. Kit for carrying out a method according to any of claims 1-19, comprising a primer pair according to any of claims 20-23 and optionally an oligonucleotide probe or a carrier, said carrier comprising at least 1 oligonucleotide probe specific for a spacer region of a bacterium to be
30 determined said bacterium not belonging to *M. tuberculosis* complex, preferably the oligonucleotide probe as defined, said oligonucleotide probe being an oligonucleotide probe of at least 10 nucleotides, preferably more than 12 nucleotides, in particular comprising between 12 to 40 nucleotides, said probe being sufficiently homologous to any of the
35 spacer sequences or to fragments or derivatives of such spacer sequences to hybridise to such a spacer sequence, said oligonucleotide probe comprising at least ten consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology, most preferably exhibiting more than

90% homology with the corresponding part of the spacer sequence.

25. Kit according to claim 24 further comprising a data carrier
with required reference patterns of the bacterial strain to be
determined.

Claims

1. A method of in vitro amplification of nucleic acid using amplification primers in a manner known per se, in amplification reactions such as PCR, LCR or NASBA, wherein a pair of primers is used comprising oligonucleotide sequences sufficiently complementary to a part of the Direct Repeat sequence of a bacterium other than a microorganism belonging to the Mycobacterium tuberculosis complex of microorganisms for hybridisation to a Direct Repeat to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction, wherein the Direct Repeat is a sequence with a length between 20-50 base pairs which occurs 5-60 times in a region of the bacterial genome, whereby the Direct Repeat sequences are separated by spacer sequences with a length of between 20-50 nucleotides, said spacer sequences being non repetitive.

2. A method according to claim 1 wherein the Direct Repeat sequence is obtainable from screening a genomic bacterial nucleic acid sequence using the programme Patscan wherein the Direct Repeat is designated p1 with a length between 20-50 basepairs then p1 is sought 20-50 basepairs downstream of p1 as the pattern

p1=(20..50)(20..50)p1(20..50)p1 or a variant thereof wherein the ranges of the nucleotide lengths are shorter and wherein the frequency of occurrence of the Direct Repeat can vary between 5 and 60.

- 5 3. A method according to claim 1 ~~of 2~~ wherein the Direct Repeat has a length between 30-40 base pairs and the spacer has a length of 35-45 base pairs.
- 10 4. A method according to claim 1 ~~any of the preceding claims~~ wherein the Direct Repeat has a terminus exhibiting at least 3 out of 5 nucleotides identical with the sequence GAAAC, preferably 4, said termini being selected from GAAAC, GAAXXC GAACTC, GXAAC, GCAAC, GAAA, GAAXC, GAAGC, AAAC.
- 15 5. A method according to claim 1 ~~any of the preceding claims~~ wherein the Direct Repeat terminates with GAACTC, ATACAC, AAAACT, TTGCAA, GGAAAC, TGAAAC, TGAAGC, TGGAAA, TTAAAC, TGAAAT or TTCAAC.
6. A method according to claim 1 ~~any of the preceding claims~~ wherein the Direct Repeat has stretches of 3-4 identical bases.
- 20 7. A method according to claim 1 ~~any of the preceding claims~~ wherein the Direct Repeat has a sequence such that it is not prone to loop formation or any other obvious secondary structure.
- 25 8. A method according to claim 1 ~~any of the preceding claims~~ wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative aerobic/microaerophilic rods and cocci and facultatively anaerobic gram-negative rods.
9. A method according to claim 8 wherein the bacterium is a pathogenic bacterium selected from the genera Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas, Bordetella, Staphylococcus, Streptococcus and Acinetobacter.

a 10. A method according to ³~~any of the preceding claims~~ claim 1, wherein said primers have oligonucleotide sequences complementary to non overlapping parts of the Direct Repeat sequence and such that the elongation reactions from each primer can occur without hindrance of the other when both primers hybridise to the same Direct Repeat
5 and undergo elongation.

a 11. A method according to ^{claim 1}~~any of the preceding claims~~, wherein one primer DRa is capable of elongation in the 5' Direction and the other primer DRb is capable of elongation in the 3' Direction and DRa is complementary to a sequence of the Direct
10 Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary.

a 12. A method of detection of a bacterium, said bacterium not belonging to the M tuberculosis complex of microorganisms said method comprising
15 1) amplifying nucleic acid from a sample with the method according to any of the preceding claims, followed by
2) carrying out a hybridisation test in a manner known per se, wherein the amplification product is hybridised to an oligonucleotide probe or a plurality of different oligonucleotide probes, each oligonucleotide being sufficiently
20 homologous to a part of a spacer of the Direct Region of the bacterium to be determined for hybridisation to occur to amplified product if such spacer nucleic acid was present in the sample prior to amplification, said hybridisation step optionally being carried out without prior electrophoresis or separation of the amplified product.
25 3) detecting any hybridised products in a manner known per se.

13. A method according to claim 12, wherein the hybridisation test is carried out using a number of oligonucleotide probes, said number comprising at least a number of oligonucleotides probes specific for the total spectrum of bacteria it is desired to detect.
30

a 14. A method according to claim 12 ~~or 13~~, wherein the oligonucleotide probe is at least ten oligonucleotides long and is a sequence complementary to a sequence selected from any of the spacer sequences of the Direct Repeat region of the bacterium to be determined or is a sequence complementary to fragments or derivatives of said spacer sequences, said oligonucleotide probe being capable of hybridising to such a spacer sequence and comprising at least ten consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology with such a spacer sequence.

10 15. A method according to claim 12 ~~any of claims 12-14~~ wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative aerobic/microaerophilic rods and cocci and facultatively anaerobic Gram-negative rods.

a 15 16. A method according to claim 12 ~~any of claims 12-15~~ wherein the bacterium is a pathogenic bacterium selected from the genera Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas and Bordetella and the group of Gram positive bacterial genera Staphylococcus and Streptococcus as target for the differentiation method.

20 17. A method for differentiating the type of bacterium in a sample, said bacterium not belonging to the M. tuberculosis complex, said method comprising carrying out the method according to claim 12 ~~any of claims 12-16~~, followed by comparison of the hybridisation pattern obtained with a reference.

25 18. A method according to claim 17, wherein the reference is the hybridisation pattern obtained with one or more known strains of the bacterium to be determined in analogous manner.

a 30 19. A method according to claim 17 ~~or 18~~ wherein the reference is a source providing a list of spacer sequences and sources thereof, such as a data bank.

20. A pair of primers wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism *E. coli* for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular a Sequence described in SEQ ID No. 1-18 and/or exhibits at least 60% homology, with the corresponding part of the Direct Repeat sequence.

21. ^{claim 20} Primer pair according to ~~claim 21~~, comprising one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of SEQ IS No. 2.

22. A pair of primers wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism *S. typhimurium* for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular the DEQ ID No. 2 and/or exhibits at least 60% homology, with the corresponding part of the Direct Repeat sequence.

23. Primer pair according to claim 22, comprising one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of SEQ ID No. 8.

24. Kit for carrying out a method according to any of claims 1-19, comprising a primer pair according to any of claims 20-23 and an oligonucleotide probe or a carrier, said carrier comprising at least 1 oligonucleotide probe specific for a spacer region of a bacterium to be determined said bacterium not belonging to *M. tuberculosis* complex, said oligonucleotide probe being an oligonucleotide probe comprising between 12 to 40 nucleotides, said probe being sufficiently homologous to any of the spacer sequences or to fragments or derivatives of such spacer sequences to hybridise to such a spacer sequence, said oligonucleotide probe comprising at least ten consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, with the corresponding part of the spacer sequence.

25. Kit according to claim 24 further comprising a data carrier with required reference patterns of the bacterial strain to be determined.